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A smart bilayer scaffold of elastin-like recombinamer and collagen for soft tissue engineering

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Abstract Elastin-like recombinamers (ELRs) are smart, protein-based polymers designed with desired peptide sequences using recombinant DNA technology. The aim of the present study was to produce improved tissue engineering scaffolds from collagen and an elastin-like protein tailored to contain the cell adhesion peptide RGD, and to investigate the structural and mechanical capacities of the resulting scaffolds (foams, fibers and foam-fiber bilayer scaffolds). The results of the scanning electron microscopy, mercury porosimetry and mechanical testing indicated that incorporation of ELR into the scaffolds improved the uniformity and continuity of the pore network, decreased the pore size (from 200 to 20 μm) and the fiber diameter (from 1.179 μm to 306 nm), broadened the pore size distribution (from 70–200 to 4–200 μm) and increased their flexibility (from 0.007 to 0.011 kPa^{-1}). Culture of human fibroblasts and epithelial cells in ELR-collagen scaffolds showed the positive contribution of ELR on proliferation of both types of cells.

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1 Introduction

Materials science has begun to take advantage of the power of new techniques in molecular biology and genetic engineering such as recombinant DNA technology, which allows the introduction of a gene in the genetic content of a microorganism, plant or other eukaryotic organisms and induce the production of its encoded protein-based polymer as a recombinant protein [1]. These kinds of macromolecules are being generically named as "recombinamers" [2]. This technology is superior to any other polymer synthesis technology in terms of the control, complexity and fine-tuning possibility that it offers. Using this technology, it is possible to bioengineer protein-based polymers (PBPs) of more complex and well-defined structure. Elastin-like recombinamers (ELRs) form a subclass of these biocompatible PBPs. They are composed of the pentapeptide repeat Val-Pro-Gly-Xaa-Gly (VPGXG), which is derived from the hydrophobic domain of tropoelastin and where X represents any natural or modified amino acid, except proline [3].

ELRs have been used as coatings [4] and films [5] for improved cell attachment, as hydrogels to promote chondrogenesis [6–8] or as polymer injections [9, 10]. They could also be shaped into fibers in pure form [11]. The first ELR candidates for tissue engineering applications were simple polymers, to which the cells did not attach. Soon after, they were enriched with short peptides having specific bioactivity [1]. Recently, a scaffold containing an ELR with substrate amino acids for mTGase, recognition sequences for endothelial cell adhesion (REDV), elastic mechanical behavior (VPGIG), and for targeting of specific

elastases for proteolytic reabsorption (VGVAPG), was found to be suitable for vascular tissue engineering [12]. To our knowledge, the present study is the first to use an ELR engineered to carry the cell adhesion peptide RGD in combination with collagen to prepare foams, fibers and a combination of them (foam-fiber bilayer structures) as potential scaffolds for soft tissue engineering. The structural and mechanical capacities of the resulting scaffolds were studied. Primary human fibroblasts and epithelial cells, isolated from non-keratinized cheek region of oral cavity, were cultured in ELR-collagen and control collagen scaffolds to study the influence of the presence of ELR on cell proliferation and tissue development.

Structural and mechanical characteristics of a scaffold are very important, since they affect cell infiltration and proliferation within the scaffold, as well as cell–cell communication and medium perfusion, and also biodegradability which should be synchronized to the reconstruction of normal tissue [13, 14]. Pore structure has been observed to significantly affect cell binding and migration in vitro and influence the rate and depth of cellular in-growth in vitro and in vivo. Additionally, scaffold mean pore size was found to significantly influence cell morphology and phenotypic expression [15]. An ideal scaffold should have similar mechanical and physical properties to the tissue it replaces [16]. Crosslinking is a major concern when using collagen and ELR to reconstruct scaffolds because both are readily soluble in aqueous media which make them unsuitable for cell culture and in vivo applications when not crosslinked. Several methods have been reported to crosslink collagen-based scaffolds, glutaraldehyde being the most common crosslinking agent in clinical use today for fixing collagenous tissues [17]. However, glutaraldehyde has been shown to be cytotoxic when used in certain concentrations and it was also reported to disrupt the fiber morphology in fibrous mats [18]. Genipin is a naturally occurring crosslinking agent derived from the fruits of *Geinpa americana* and *Gardenia jasminoides* Ellis and has proven to be an effective crosslinker for proteinaceous tissues [17]. It has been shown to be less toxic than even carbodiimide crosslinking which is considered as a non-toxic chemical crosslinking method [19]. To determine an effective crosslinker for ELR-collagen hybrid foams and fibrous mats, two non-toxic methods: genipin crosslinking, a chemical method, and a dehydrothermal treatment (DHT), a physical crosslinking method which introduces covalent crosslinks between the polypeptide chains of the collagen fibers at high temperatures and under vacuum, were applied and compared. It was reported in the literature that tissue in-growth into collagen-based materials that have been crosslinked by dehydrothermal methods occurs to a much greater extent than that which follows glutaraldehyde treatment, and that for tissue engineering applications, where tissue and cellular in-growth is desirable, methods such as dehydrothermal treatment as opposed to glutaraldehyde could be employed for collagen-based scaffold stabilization [20].

2 Materials and methods

2.1 Scaffold preparation

All collagen-elastin-like recombinamer scaffolds were prepared with a ratio of 3:1 (w/w).

2.2 Collagen type I isolation

Collagen type I was isolated from Sprague–Dawley rat tails as reported earlier [21]. Briefly, the tails were dissected and tendons were placed in cold acetic acid (0.5 M, Merck, USA). The solution was then filtered, dialyzed against sodium phosphate buffer (pH: 7.2), precipitated and centrifuged. Sodium chloride was added (5%) and the procedure was repeated. The final pellet was sterilized in 70% ethanol (Merck, USA), frozen and lyophilized (Labconco Corp., USA). Purity of the collagen was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 H-RGD6 expression and purification

The featured ELR contains 6 monomers of RGD, a histidine-tag, 6 aspartic acids, 24 lysines and 7 histidines, which are charged residues, being designated as H-RGD6 (Fig. 1).

Expression conditions and purification protocols were adapted from McPherson et al. [22] and Girotti et al. [23], as reported earlier [24]. Gene expression of a recombinant *Escherichia coli* strain BLR (DE3) containing the expressing gene of H-RGD6 was induced in a 12 L Applikon fermentor, in terrific broth medium (TB) with 0.1% of carbenicilin and 0.1% of glucose, under controlled conditions of temperature (37°C) and pH (7.00). The fermentation was stopped after registering an optical density variation, at 600 nm (OD600) inferior to 0.25, in a time frame of 1 h. Subsequent to fermentation, the culture was harvested by centrifugation, resuspended and lysed by ultrasonic disruption. Insoluble debris was removed by centrifugation and the cleared lysate was subjected to

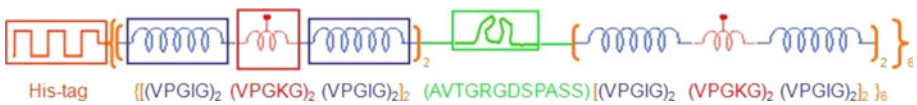

Fig. 1 Schematics of the featured elastin-like recombinamer, H-RGD6

several cycles of cold and warm centrifugations, of 4 and 40°C, respectively. All the purification steps were carried out in a sodium chloride (NaCl) solution at 0.5 M. The polymer solution was then frozen at −24°C and lyophilized.

2.4 H-RGD6 characterization

2.4.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed to assess H-RGD6 purity after purification. For this test, 5 μl of a H-RGD6 solution at 1 mg/ml were loaded in a polyacrylamide gel. The percentages of the acrylamide/bisacrylamide (30% stock) in 12% separating gel (pH 8.8) and stacking gel (pH 6.8) were 40 and 13%, respectively. The identification of an intense band around 60 kDa was expected to confirm the presence of the polymer and its purity.

2.4.2 Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy

To further assess H-RGD6 purity and molecular weight, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectroscopy was performed in a Voyager STR, from Applied Biosystems, in linear mode and with an external calibration using bovine serum albumin (BSA).

2.4.3 Aggregate size measurements

H-RGD6 aggregate size in solution was measured using a Nano-ZS from Malvern (United Kingdom), for a range of temperatures between 25 and 40°C, with a stabilization time of 5 min. H-RGD6 samples were prepared at 1 mg/ml in phosphate buffer solution (PBS, pH 7.4). 12 runs were performed for each sample to determine the particle/aggregate size, in order to obtain a final average value at constant temperature.

2.5 Preparation of ELR-collagen foams

Porous ELR containing collagen scaffolds were prepared by either incorporating the ELR into the scaffold by mixing its solution with collagen solution, or by adsorbing it onto the surface of the collagen foams. To incorporate it within the scaffold, collagen and ELR were dissolved in acetic acid (0.5 M) and phosphate buffered saline (PBS, pH 7.2), respectively (both 13.5 mg/ml) and three volumes of collagen solution were vigorously mixed at room temperature with one volume of ELR solution so that the final total protein concentration was 1.35%. The mixture was then added to 24 well plates, frozen at −20°C for 24 h and lyophilized for 13 h. To adsorb the ELR onto collagen foam surface, pure collagen foams were prepared by dissolving collagen (1.35%) in acetic acid (0.5 M), then lyophilizing as earlier. The resulting foams were then incubated with the ELR solution (0.1% in PBS) at 37°C for 2 h. Collagen foams (1.35%) without ELR were prepared as controls.

To obtain closed surfaced foams with a compact skin layer, 10% ethanol (v/v) was present in the protein solution during foam preparation and the foams were frozen at −80°C for 24 h prior to lyophilization, instead of −20°C, to further close the pores on the surface. Addition of ethanol in foam solution before lyophilization was previously shown to result in closed surfaced scaffolds, and lower freezing temperatures in smaller pores [25].

2.6 Preparation of ELR-collagen fibrous mats

ELR containing fibrous mats were prepared via electrospinning collagen-ELR and pure collagen solutions to investigate the influence of ELR incorporation on scaffold properties. Collagen (9.3%) and ELR (13.2%) were dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP, Sigma-Aldrich, USA) and PBS (pH 7.2), respectively; and four volumes of collagen solution were mixed with one volume of ELR solution so that the final total protein concentration was 10% and the collagen/ELR ratio was always 3:1 (w/w) as in foams. Pure collagen fibrous mats, having the same protein concentration (10%), were also prepared as controls. Electrospinning was performed by loading the solution in a 10 ml syringe fitted with a 3 cm 18G blunt-end needle (Ayset AS, Turkey) and dispensing at a rate of 20 μl/min. The solution was charged by applying a voltage of 22.5 kV and the fibers were collected on a flat, grounded plate covered with aluminum foil placed at a distance of 10 cm from the syringe needle.

2.7 Preparation of ELR-collagen foam-fiber bilayer scaffolds

ELR containing foam-fiber bilayer scaffolds were prepared by electrospinning the previously described ELR-collagen solutions directly onto foams which were taped slightly on the grounded collector. In this way, the upper part of the resulting bilayer scaffold was fibrous and the lower part was porous. The fibers were collected onto two types of foams: porous and closed surfaced.

2.8 Crosslinking of the scaffolds

Collagen-ELR and pure collagen scaffolds were crosslinked chemically by using genipin (Wako Chemicals, Germany), physically via dehydrothermal treatment (DHT)

or by the combination of both. For genipin crosslinking, genipin was dissolved in either 70% ethanol as commonly reported in literature, or in PBS (due to the possibility that ethanol might cause the surface of the foams to become closed). Genipin solution was then added to the collagen-ELR solution with vigorous mixing to have a final concentration of 0.1% w/v and the final mixture was incubated at room temperature (RT) for 48 h before lyophilization. Addition of the crosslinker before lyophilization, instead of afterwards, was reported to better conserve the foam structure [26]. To crosslink fibrous mats, genipin was either added to the collagen solution prior to electrospinning, or afterwards (0.1% w/v, 48 h, RT). For DHT crosslinking, foams and fibrous mats were incubated at elevated temperatures under 685.8 mmHg pressure. Different temperatures and incubation times (105°C for 24 h, 105°C for 72 h, 150°C for 24 h, 150°C for 48 h) were tested. The two methods were also combined; DHT (150°C, 24 h) followed by genipin crosslinking (0.1%, 48 h, RT). To determine whether the scaffolds would maintain their integrity during tissue culture, crosslinked scaffolds were incubated in PBS at 37°C and observed for over 1 month.

2.9 Scaffold Characterization

2.9.1 Foam thickness

Thicknesses of the scaffolds were measured using a standard micrometer with a sensitivity of 0.1 μm.

2.9.2 Pore size and its distribution

Pore size and its distribution in the foams were determined with a mercury porosimeter (Poremaster 60; Quantachrome) under low pressure (50 psi) at METU Central Lab.

2.9.3 Morphology and microstructure of scaffolds

Morphology, the surface and inner microstructures of the foams and fibrous scaffolds were studied with a FEI Quanta 200F (USA) scanning electron microscope (SEM) at UNAM (Bilkent Univ.) after fracturing sections and sputter-coating (12 nm) with gold–palladium. Fiber diameters and pore sizes were measured using the image processing program NIH ImageJ using 4 different areas of the image to calculate the average values. The results were expressed as mean ± standard error of the mean (SEM).

2.9.4 Mechanical properties

A mechanical testing system (LRX 5K, Lloyd Instruments Ltd, UK), controlled by a computer program (WindapR), was used to perform compression tests on the scaffolds. The cell load was 5 kN and the crosshead displacement rate was 10 mm/min. Elastic modulus (E*), flexibility (1/E*), elastic collapse stress and strain (σ_{el}*, ε_{el}*), and the collapse plateau modulus ($\Delta\sigma/\Delta\varepsilon$) of the scaffolds were calculated from the load-strain graphs as previously described for low-density, open-cell foams [27].

2.10 In vitro studies

2.10.1 Origin, isolation, and culture of fibroblasts and epithelial cells

Fibroblasts and epithelial cells were isolated from human oral mucosal biopsies removed from the nonkeratinized cheek region of the mouth, and obtained with informed consent from patients undergoing oral surgery, as previously reported [28]. Briefly, the separation of the epithelium from the lamina propria was performed with dispase (GIBCO), 10 mg/ml for 3 h at 4°C. After separation, epithelium was treated with trypsin 0.5 g/l—EDTA 0.2 g/l for 20 min to extract epithelial cells, which were grown at 8,000 cells/cm^2 on a feeder layer of irradiated human fibroblasts in a specially designed medium as follows: DMEM-Ham-F12 2.78/1 (Sigma), 10% fetal calf serum (Hyclone), 0.4 μg/ml hydrocortisone (Upjohn), 0.12 UI/ml insulin (Umuline, Lilly), 0.033 μg/ml selenium (Laboratoire Aguettant), 0.4 μg/ml isoprenaline hydrochloride (Isuprel, Sterling Winthrop), 2×10^{-9} M, triiodo thyronine (Sigma), 10 ng/ml epidermal growth factor (Austral Biologicals), and antibiotics. After epithelium-lamina propria separation, fibroblast isolation was performed with collagenase A (Roche Diagnostics), 0.1 U/ml for 20 min at 37°C with continuous stirring. The digest was purified using a 70 μm cell strainer (BD Biosciences). This procedure was repeated 6 times, and then the digest was immediately placed in monolayer culture. Fibroblasts were seeded at a density of 10,000 cells/cm^2 and cultured in fibroblast medium composed of DMEM, 10% newborn calf serum (NCS) (Hyclone, France), and antibiotics (Penicillin G 100 UI/ml and Streptomycin 100 μg/ml, Panpharma, France). Fibroblasts and epithelial cells were seeded on the foams at passage 3.

2.10.2 Culture of fibroblasts and epithelial cells in ELR-collagen and control collagen foams

A suspension of 2.5×10^5 fibroblasts/cm^2 was added on top of the 1 cm^2 ELR-collagen and control collagen foams. They were then cultured for 21 days in a medium composed of DMEM, 10% fetal calf serum, 10 ng/ml epidermal growth factor, 50 μg/ml ascorbic acid (Bayer), to obtain lamina propria equivalents. Culture medium was changed daily until the seeding of epithelial cells. Human

epithelial cells were plated on lamina propria equivalents at a concentration of $2.5 \times 10^5/cm^2$. Epithelialized oral mucosal substitutes were cultured in epithelial cell medium supplemented with 50 μg/ml ascorbic acid (Bayer) under submerged conditions for 7 days. They were then elevated at the air–liquid interface for the remaining 14 days in another medium with DMEM-Ham-F12 2.2/1 (Sigma), 8 mg/ml bovine serum albumin, 0.4 μg/ml hydrocortisone (Upjohn), 0.12 UI/ml insulin (Umuline, Lilly), 50 μg/ml ascorbic acid (Bayer), and antibiotics to give rise to full-thickness oral mucosal equivalents.

2.10.3 Immunohistochemistry

The full-thickness oral mucosal equivalents based on ELR-collagen and control collagen scaffolds were immunolabelled with the antibody anti-cytokeratin 13 (Chemicon, USA), the major differentiation marker of non-keratinized human oral mucosa epithelium, and the cell nuclei were counterstained with propidium iodide (Vector Laboratories, USA). For the detection of keratin 13, tissue equivalents were embedded in OCT and frozen at −20°C. Then, sections of 6 μm thickness were fixed in acetone for 10 min at −20°C and blocked in phosphate buffered saline containing 4% bovine serum albumin and 5% normal goat serum. The primary antibody was incubated for 90 min at room temperature. The secondary antibody was AlexaFluor 488 IgG (Invitrogen, USA). Specimens were analyzed with a Nikon Eclipse Fluorescence Microscope.

3 Results

3.1 Characterization and purity of collagen type I isolated from rat tails

Purity of the isolated type I collagen was confirmed by SDS-PAGE (Fig. 2). Column 1 shows the protein marker, where the bands are at 260, 140, 100 and 70 kDa (top to bottom). The isolated collagen (RTC, column 2) had doublets at apparent molecular weights of 115 and 130 kDa, and at 215 and 235 kDa which is the typical band pattern for type I collagen. The absence of any other bands indicates that the collagen isolated from rat tails is pure type I collagen.

3.2 Production and stimuli-responsive behaviour of H-RGD6

The bioproduced polymer, labeled H-RGD6, has the structure described in Fig. 1. Through the described protocol, a bioproduction yield of around 200 mg/l was achieved after purification. ELRs are soluble in water below their transition temperature (T_t) and segregate from the solution above that temperature. This temperature-responsive behavior has been exploited to purify the polymer from the bacterial lysate [22]. Figure 3 shows the results of the MALDI-TOF and SDS-PAGE tests. These tests were performed to verify the purity of the sample and correctness of the molecular weight of H-RGD6 after expression and purification protocols.

The experimental molecular weights found by both techniques match well with the theoretical molecular weight of the polymer (60,661 Da). MALDI-TOF spectrum shows a high intensity peak at 60,543 Da, which is approximate to the theoretical value of H-RGD6. SDS-PAGE also permits to identify an intense band corresponding to the biopolymer, with the same molecular weight. In conclusion, the characterization tests point to the bioproduction of a polymer with the desired composition, sequence, molecular weight and purity.

The stimuli-responsive nature of H-RGD6 in PBS (pH 7.4) was studied by measuring the temperature dependence of the aggregate size of the biopolymer chains. The results were plotted in Fig. 4 as a function of temperature, and reflect the segregation of ELRs and formation of larger aggregates in suspension above T_t, which causes an abrupt increase in turbidity.

The aggregate size measurement indicated a T_t around 32°C in this particular solution. Across this temperature,

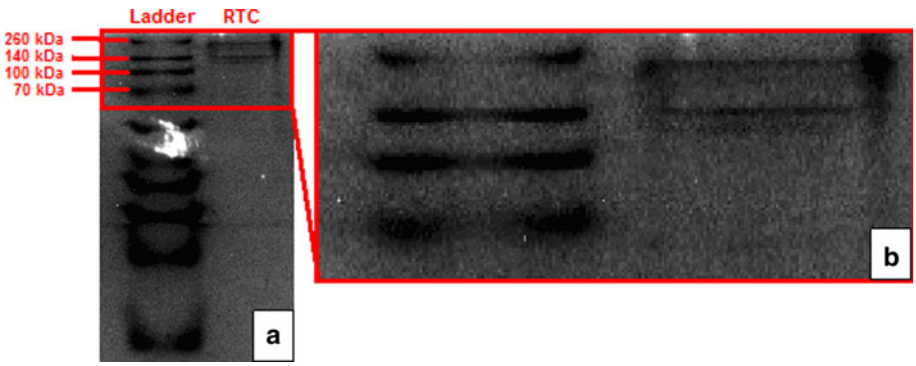

Fig. 2 Purity of collagen isolated from rat tail tendons. **a** SDS-PAGE analysis of type I collagen isolated from rat tail tendons. *First column* shows the protein marker (ladder) and *second column* the isolated rat tail collagen (RTC). **b** Magnified version of **a**

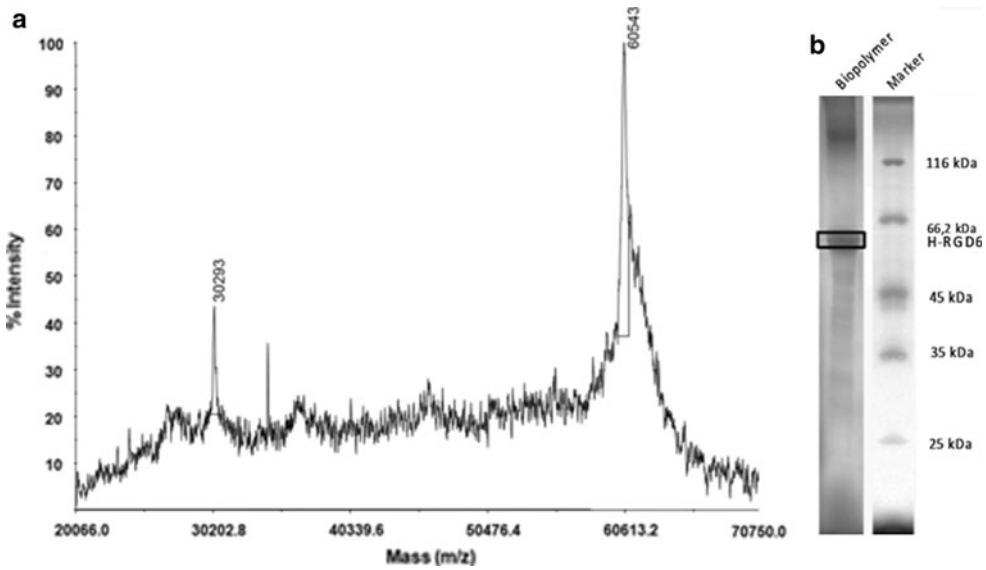

Fig. 3 Assessment of H-RGD6 purity and molecular weight. The expected mass of the polypeptide was 60,611 Da. **a** MALDI-TOF of the biopolymer. Signal at 30,293 Da is assigned to doubly charged species. **b** Analysis of biopolymer extract by SDS-PAGE

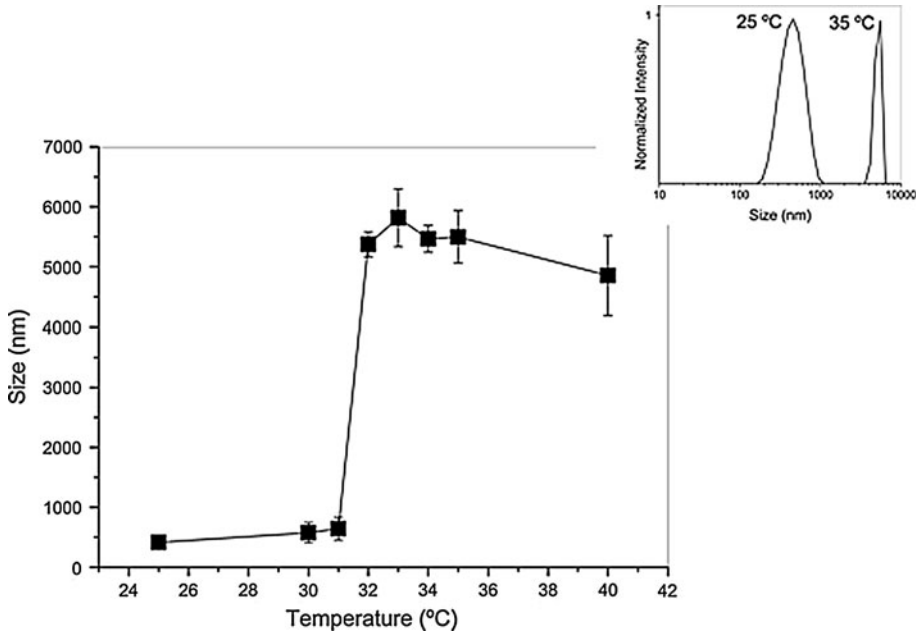

Fig. 4 Aggregate size profile for a 1 mg/ml H-RGD6 solution in PBS (pH 7.4) in the temperature range 25–40°C. *Error bars* represent one standard deviation. The *inset* graphics present representative size distribution profiles at temperatures below and above the T_t

an increase in the aggregate size from around 650 to 5,400 nm was found, which was probably due to the hydrophobic association of the free chains of the biopolymer and consequent increase of aggregate size. Figure 4 also shows the representative size distribution at 25 and 40°C, indicating a quite narrow and monomodal distribution of the size of the objects in solution below and above the T_t.

3.3 Structural properties of the scaffolds

Structural properties of all scaffolds (foams and fibers) depended strongly on ELR presence and the crosslinking method. When ELR was adsorbed onto the collagen foam surface, the resulting scaffold had semi-closed pores on the surface (Fig. 5e); whereas, when it was mixed with collagen solution before lyophilization, the resulting scaffold had open, porous surface (Fig. 5c, d). When pure and ELR incorporated collagen foams were compared (Fig. 5a, c), the former had thicker pore walls and larger pore size; whereas, the latter had thinner pore walls and smaller pores (Fig. 5; Table 1). The pore size distribution was broaden in ELR containing foams (Table 1).

Genipin crosslinking significantly decreased the pore size; whereas, DHT did not affect the pore size, or its distribution (Figs. 5, 6; Table 1). DHT crosslinked scaffolds had highly porous surface, bottom and inner microstructure (Fig. 6).

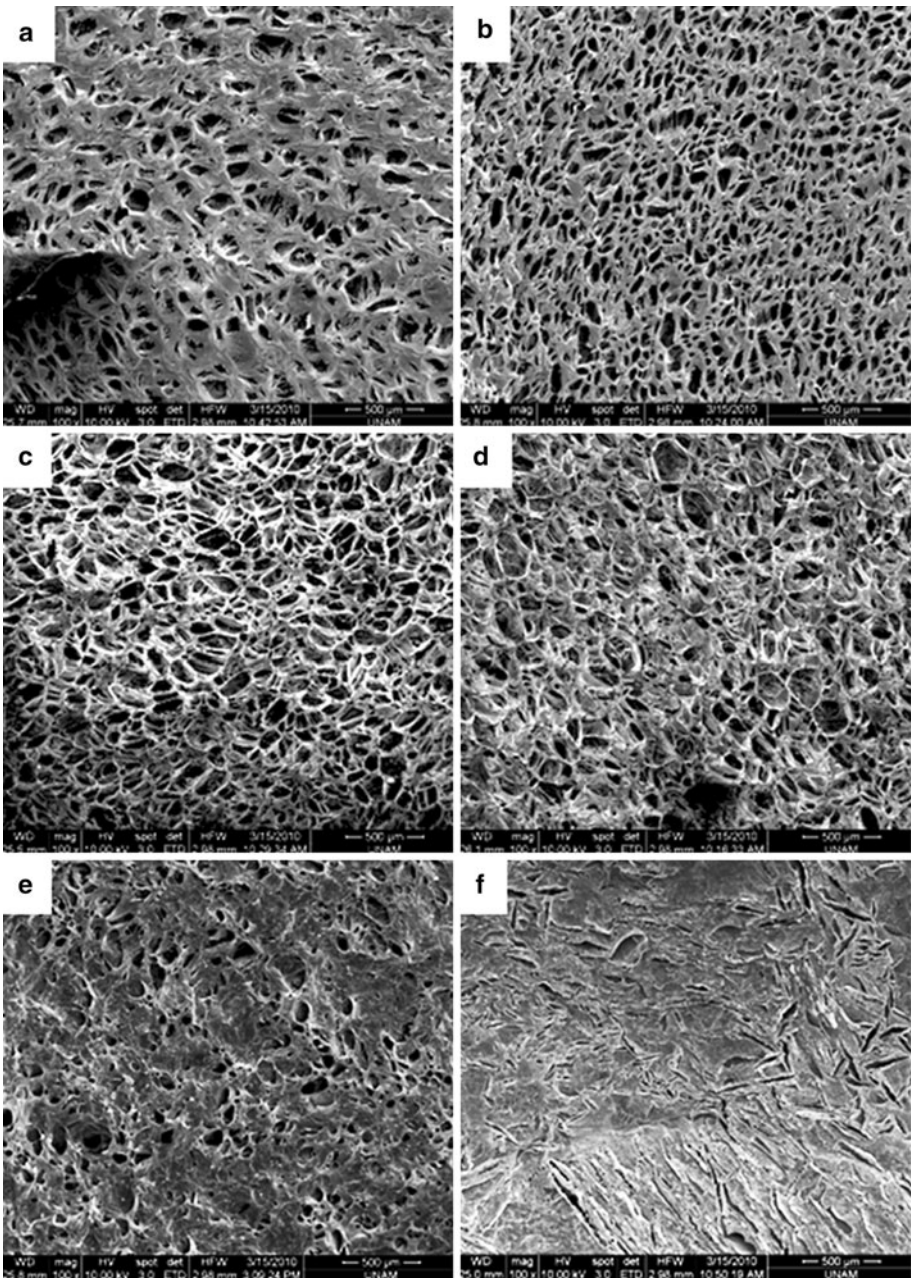

Fig. 5 SEM micrographs of **a** uncrosslinked collagen foam, **b** DHT crosslinked (150°C, 48 h) collagen foam, **c** uncrosslinked ELR-collagen foam, **d** DHT crosslinked (150°C, 48 h) ELR-collagen foam, **e** collagen foam with ELR adsorbed on its surface, **f** collagen foam prepared with ethanol addition (10% v/v)

Table 1 Thickness, pore size and pore size distribution of collagen and ELR-collagen foams dependent on crosslinking

Scaffold	Thickness (mm)	Pore size (μm)[a]	Pore size distribution (μm)
Uncrosslinked collagen foam	5.00	200	70–200
Uncrosslinked collagen + ELR foam	4.00	20	4–200
DHT crosslinked collagen foam	5.30	200	80–200
DHT crosslinked collagen + ELR foam	4.30	20	4–200
GP crosslinked collagen foam	4.67	30	4–200
GP crosslinked collagen + ELR foam	3.83	15	4–200

[a] The most abundant pore size in a foam, obtained by porosimetry

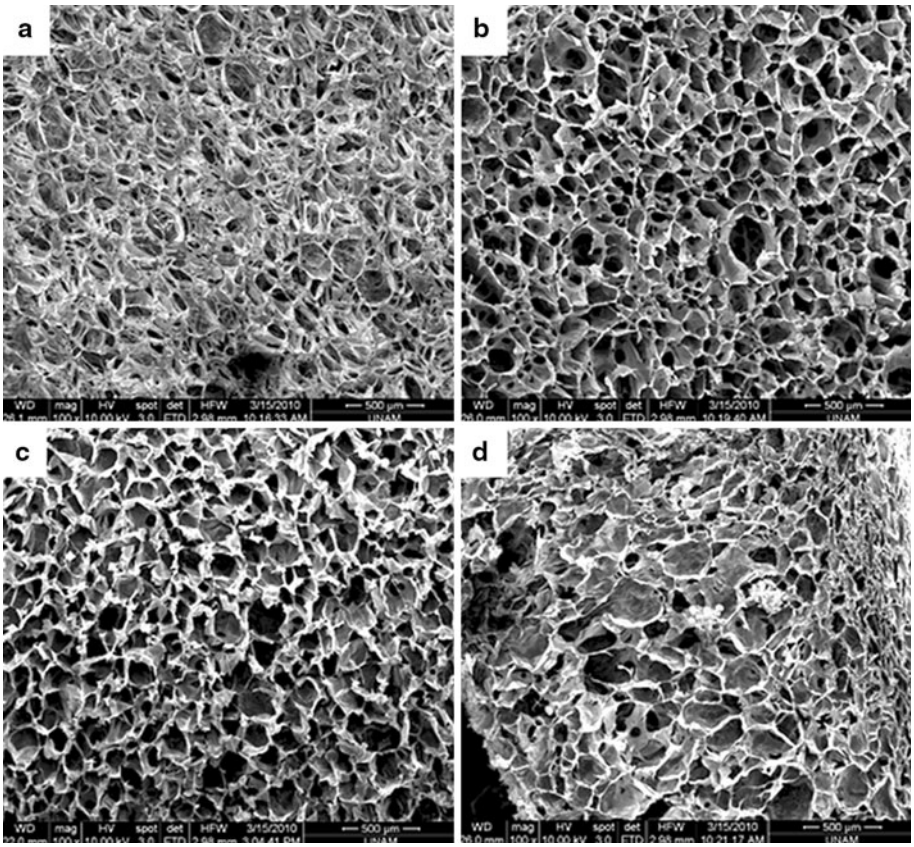

Fig. 6 SEM analysis of **a** the surface, **b** the inner middle plane, **c** the bottom and **d** the cross section of the porous collagen-ELR foams crosslinked via DHT (150°C, 48 h)

Addition of ethanol (10% v/v) into the ELR-collagen or pure collagen solution resulted in closed surface foams (Fig. 5f). Similarly, when genipin was dissolved in 70% ethanol and added to the protein solution, the resulting foam had closed surface as opposed to the open surfaced one prepared using genipin in PBS (Fig. 7).

Incorporation of ELR decreased the diameter of the collagen fibers significantly, from micron to nanoscale. DHT was also found to cause a decrease in the diameter of both pure collagen and ELR-collagen fibers, but this reduction was not as large as the one caused by ELR (Fig. 8). Genipin crosslinking destroyed the fibrous structure, regardless of whether genipin was added to the protein solution prior to electrospinning or afterwards (Fig. 9). On the other hand, all dehydrothermal treatments (105°C for 24 h, 105°C for 72 h, 150°C for 24 h, 150°C for 48 h) appeared to preserve the fibrous structure (Fig. 10). However, after 24 h incubation in distilled water, only fibers treated at 150°C for 48 h or by the combination of two methods: DHT (150°C, 24 h) followed by genipin crosslinking (0.1%, 48 h, RT) persisted (Fig. 10).

To obtain bilayer scaffolds, protein solution was electrospun directly onto foams surfaces of which were either closed or open pore type (Fig. 11a). In these bilayer constructs, the upper fibrous layer was either separated from the lower spongy part by a closed pore layer (Fig. 11b), or the two parts were in connection with each other through pores (Fig. 11c). In the former, although the surface of the foam was totally closed, the inner microstructure was preserved and the inner pores were flattened and had lamellar structure (Fig. 11b). Thus, it formed a two-compartment sponge with individual fibrous and macroporous regions.

3.4 Mechanical properties of the scaffolds

Except for closed surfaced foams, all pure and ELR containing collagen foams exhibited stress–strain behavior characteristics of low density, open cell foams with distinct linear elastic, collapse plateau and densification regimes. Both ELR incorporation and crosslinking affected the mechanical properties. ELR incorporation decreased the compressive strength (σ_{el}*) and stiffness (E*) of both uncrosslinked and crosslinked (genipin and DHT) scaffolds (Table 2). On the other hand, crosslinking increased the stiffness and compressive strength of both pure and ELR containing scaffolds, where the effect of crosslinking with genipin was more pronounced than that with DHT.

3.5 In vitro studies

Before proceeding to use the ELR-collagen bilayer scaffolds for the reconstruction of full-thickness oral mucosa

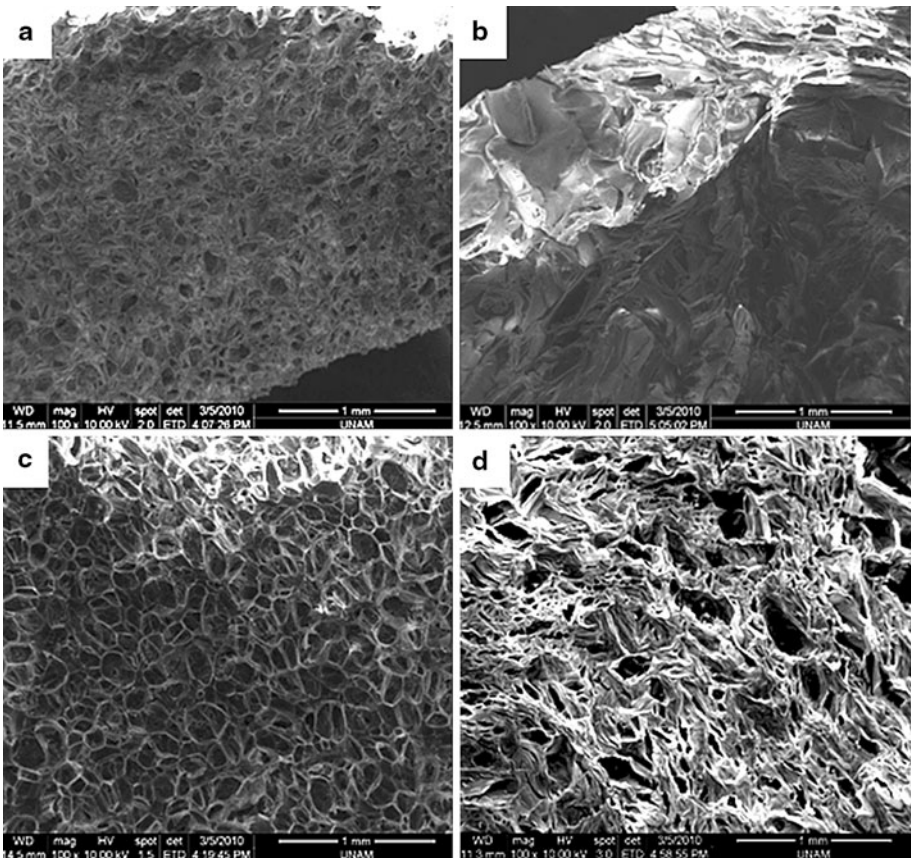

Fig. 7 SEM micrographs of collagen foams crosslinked by genipin dissolved in **a** PBS or **b** 70% ethanol, and ELR-collagen foams crosslinked by genipin dissolved in **c** PBS or **d** 70% ethanol

a	Scaffold	Mean Fiber Diameter (μm) ± SEM
	Uncrosslinked pure collagen (10%)	1.179 ± 0.094
	Uncrosslinked collagen+ELR (10%)	0.306 ± 0.025
	DHT crosslinked pure collagen (10%)	1.007 ± 0.093
	DHT crosslinked collagen+ELR (10%)	0.263 ± 0.023

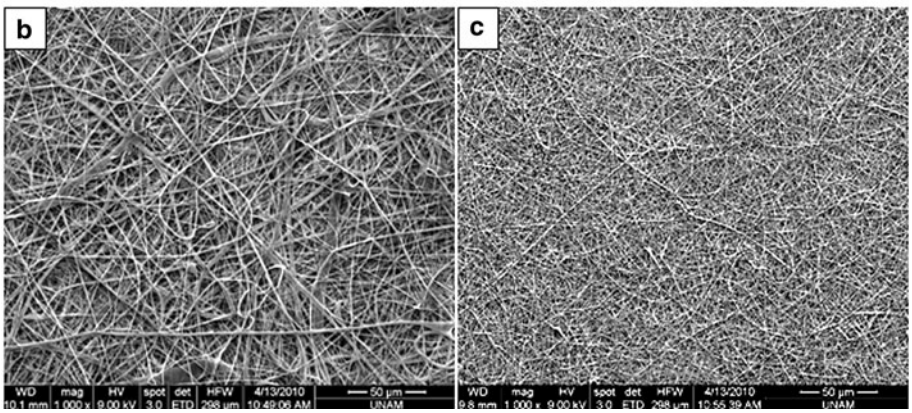

Fig. 8 Uncrosslinked and DHT crosslinked collagen and collagen-ELR (3:1, w/w) fibrous mats. **a** Mean fiber diameters ± standard error of the mean (SEM). **b** Pure collagen fibers, collagen concentration 10% (×1,000). **c** Collagen-ELR (3:1) fibers, the same total protein concentration 10% (×1,000)

and skin equivalents, the hypothesis that ELR would bring more biocompatibility to the scaffolds was tested by culturing human oral fibroblasts and epithelial cells in ELR-collagen foams and control collagen foams. The results of the immunolabelling of keratin 13 and the counterstaining of the cell nuclei with propidium iodide showed that at the end of a 6 week culture period, fibroblasts in the ELR-collagen foams migrated through the whole thickness of the scaffold, proliferated and populated the foam. The epithelium developed by the epithelial cells at the top of this scaffold was stratified and thick (Fig. 12a). On the other hand, in the control collagen foam, fibroblasts were much less in number, could not populate the scaffold, and were detected mostly at the subsurface part of the foam.

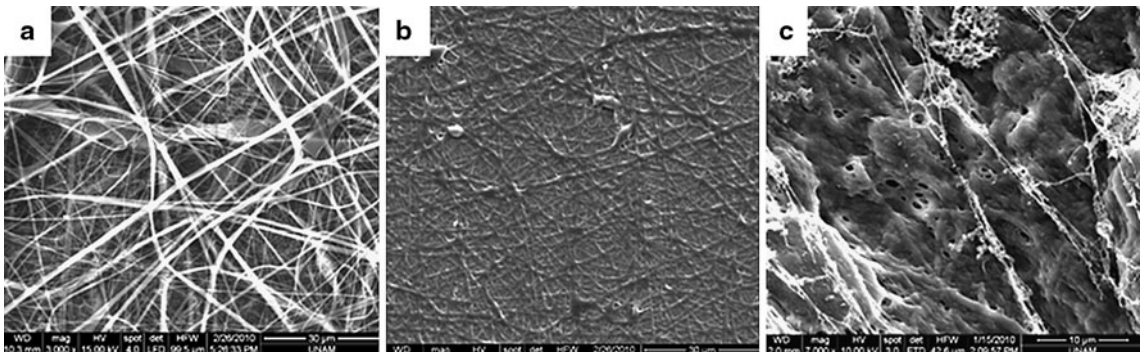

Fig. 9 Collagen fibers **a** uncrosslinked, **b** crosslinked using genipin by adding into collagen solution before electrospinning, **c** crosslinked by incubating the electrospun fibers in genipin solution (0.1 w/v, 48 h, RT)

The epithelium developed at the surface of the control collagen foam was much thinner compared to the one developed on the collagen-ELR foam (Fig. 12b).

4 Discussion

In soft tissues, collagen and elastin are the predominant components of the extracellular matrix [29]. In this study, to mimic this natural structure and at the same time to improve the conventional scaffolds made of collagen, an elastin-like recombinamer was bioengineered to contain the cell adhesion peptide RGD and was blended with collagen to produce foams, fibers and a combination of them (foam-fiber bilayer structures). In the bilayer structure, the protein solution was electrospun onto two types of foams with surfaces with closed or open pores. In the former, the fibrous top part was literally separated from the spongy bottom part by a closed pore skin layer. In this structure, the foam part was expected to serve as a support for the fragile fibers and to facilitate their handling during culture. The closed pore surface would prevent the cells seeded on the fibers from migrating into the foam. On the other hand, in the latter bilayer structure, the fibrous part is attached to the foam with open pores; this was expected to make this structure suitable for coculture studies where different cell types on different sides of the construct could communicate with each other more freely. For instance, in skin tissue engineering, as well as in oral mucosa and cornea, epithelial cells and mesenchymal cells should reside in two distinct regions: a relatively thin epithelium and a thicker extracellular matrix, but should nevertheless be able to communicate with each other for proper epithelial development [28].

Incorporation of ELR was found to have a profound effect on the structural and mechanical properties of both the foams and the fibrous mats. In foams, the pore size was significantly decreased upon incorporation of ELR whether the collagen and ELR were uncrosslinked, or dehydrothermally or chemically crosslinked with genipin. ELR also made the pore size distribution broaden, causing smaller pores to form. This drastic decrease in pore size (from 200 to 20 μm) and increase in its distribution (from 70–200 to 4–200 μm) might be beneficial in tissue culture, considering that the size of a human dermal fibroblast, the major cell type of the dermis, varies between 10 and 100 μm [30] and that fibroblast migration decreases as scaffold pore size increases above 90 μm [14]. It was also reported that scaffolds used for studies of skin regeneration were inactive when the mean pore size was either lower than 20 μm or higher than 120 μm [31]. Therefore, incorporation of ELR decreased the pore size to a suitable range for cell migration. When the ELR was added after the collagen foam was formed, instead of before, the surface of the foam was rather closed, probably because the ELR filled the pores on the surface during adsorption. Therefore, when, not only cell attachment to the surface, but also the population of the foam by the cells is important, incorporation of ELR in the foam before it is formed should be the approach. In fibrous mats, ELR led to significantly smaller diameters (>threefold, a decrease from micron to nanoscale) even when the same total protein concentration (10%) was used. ELR was previously shown to bind to collagen, and is thought that it might interfere with incorporation of more collagen molecules into the fibers [12].

Except for foams with closed pores, all pure and ELR containing collagen foams under compression exhibited stress–strain behavior that is characteristic of low density, open cell foams with distinct linear elastic, collapse plateau and densification regimes. Incorporation of ELR decreased the stiffness and compressive strength, but increased the flexibility. Therefore, pure collagen scaffolds might be advantageous in applications requiring higher strength, such as hard tissue engineering, but for soft tissue engineering, the elasticity that the ELR brings to scaffold is very appropriate.

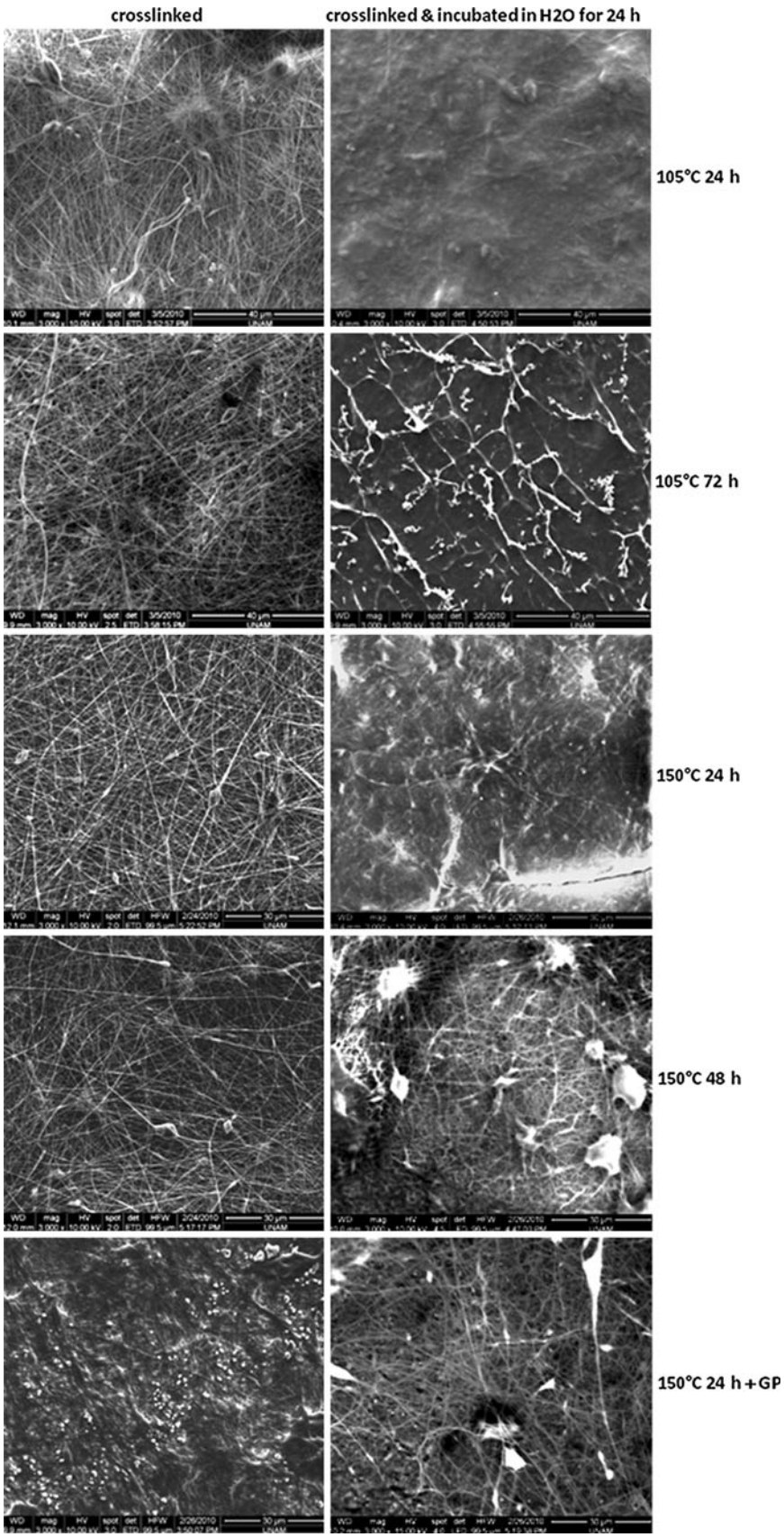

Fig. 10 Dehydrothermal treatment applied at different temperatures and periods to crosslink electrospun collagen fibers. *GP* genipin (0.1 w/v, 48 h, RT)

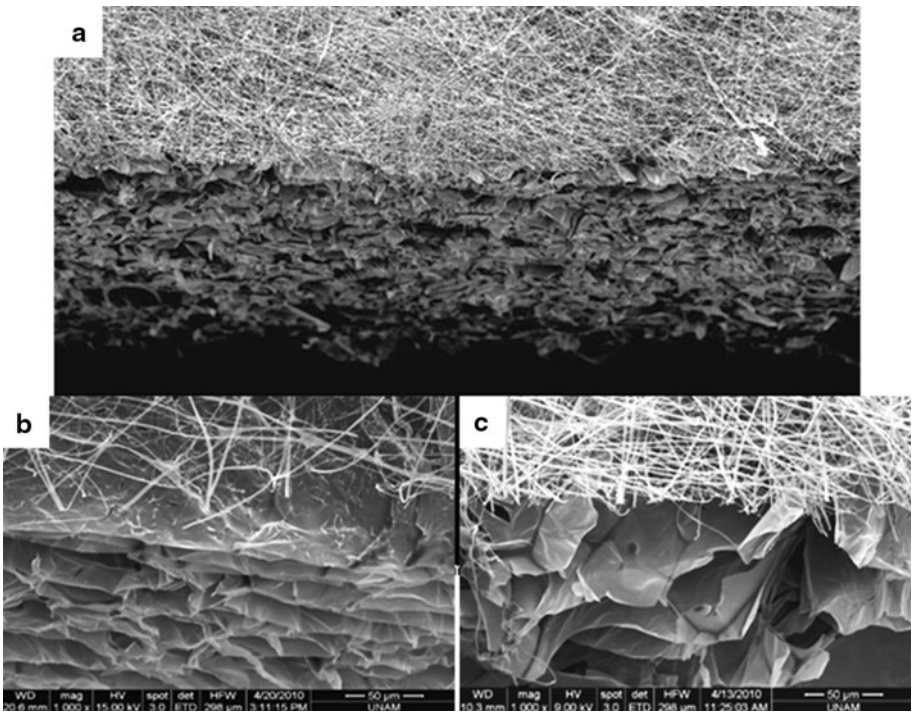

Fig. 11 SEM micrographs of foam-fiber bilayer scaffolds. **a** The upper layer of the scaffold is fibrous, while the lower spongy part is porous. **b** Fibers residing on closed surfaced foam; porous inner structure was retained although the pores were flattened and lamellar. **c** Fibers residing on open surfaced foam

Table 2 Results of compression tests on pure and ELR incorporated collagen scaffold variants including mechanical properties dependent on crosslinking

Scaffold	E* (kPa)	1/E* (kPa^{-1})	σ_{el}* (kPa)	ε_{el}*	$\Delta\sigma/\Delta\varepsilon$ (kPa)
Uncrosslinked collagen foam	139.5	0.007	29.5	0.341	53.7
Uncrosslinked collagen + ELR foam	87.3	0.011	13.5	0.272	47.0
DHT crosslinked collagen foam	155.6	0.006	44.2	0.367	77.7
DHT crosslinked collagen + ELR foam	115.0	0.008	39.8	0.327	63.0
Genipin crosslinked collagen foam	385.8	0.002	72.1	0.284	130.3
Genipin crosslinked collagen + ELR foam	140.7	0.007	42.5	0.399	47.4

E*, linear elastic modulus; 1/E*, flexibility; σ_{el}*, elastic collapse stress; ε_{el}*, elastic collapse strain; $\Delta\sigma/\Delta\varepsilon$, collapse plateau modulus

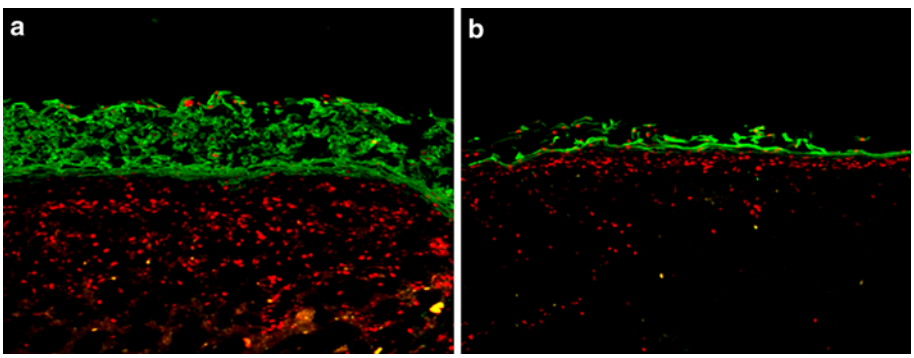

Fig. 12 Immunofluorescence labelling of keratin 13, the major differentiation marker of nonkeratinized oral mucosa epithelium, in **a** ELR-collagen foam (×100), and **b** control collagen foam (×100). Immunolabelling is shown in *green*, cell nuclei are shown in *red* (Color figure online)

In order to find an effective crosslinking method for ELR-collagen structures, genipin and dehydrothermal treatments, both non-toxic, were tested. Both scaffolds had good physical stability; remained insoluble for over 1 month in PBS at 37°C. When genipin was introduced before foam or fiber formation, foams preserved their form better (data not shown); otherwise, they tended to deform and dissolve during incubation. When compared to DHT, genipin crosslinking significantly decreased the pore size and the flexibility of both pure and ELR containing

collagen foams, but it significantly increased their compressive strength. Therefore, for hard tissue engineering, where compressive strength is crucial, genipin crosslinking should be preferred over DHT, or combined.

As has been reported in the literature, following crosslinking collagen with genipin, the normally opaque collagen turns blue with a strong fluorescence at 630 nm when excited at 590 nm [32]. With ELR the color was even darker. DHT, on the other hand, did not lead to any color changes. It is possible that in the (immuno)fluorescence analyses of tissue equivalents, the genipin-induced strong autofluorescence of the ELR-collagen scaffolds caused problems interfering with the specific fluorescent staining.

Crosslinking of ELR-collagen fibrous mats with genipin destroyed their fibrous nature, probably by dissolving them. Uncrosslinked collagen and ELR readily dissolve in aqueous media; therefore, crosslinking in an aqueous medium was not practical. Crosslinking of collagen fibers by glutaraldehyde vapor was reported to also disrupt the fibrous structure [33], although not as extensively as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) [33] or genipin (present study). In this study, DHT was used to crosslink fibers at several temperatures and periods, and it was found out that when the fibers were incubated at 150°C for 48 h or for 24 h followed by genipin crosslinking (0.1 w/v in PBS, 48 h, RT), they preserved the fibrous structure of the proteins even after 24 h incubation in distilled water. When uncrosslinked and crosslinked fibers were compared, it was found that DHT crosslinking slightly; decreased the diameter of both pure collagen and ELR-collagen fibers as opposed to glutaraldehyde crosslinking, which caused an increase in collagen fiber diameter [34].

Scaffolds act both as physical support structures and as regulators of cell activity. Microstructural and mechanical properties of scaffolds have been shown to significantly affect cell behavior such as adhesion, growth, and differentiation, and to influence the bioactivity of scaffolds used for in vivo regeneration applications of various tissues, such as cartilage, skin, and peripheral nerves [14]. Here, the foam-fiber bilayer scaffolds designed using ELR and collagen, and crosslinked by genipin or DHT were assessed for their microstructural and mechanical capacities. DHT crosslinked structures proved their potential for use in soft tissue engineering due to their continuous and interconnected pore network, suitable pore size and its distribution, neat fibrous structure, high flexibility and adequate mechanical stability. The presence of ELR was found to increase the proliferation of both fibroblasts and epithelial cells, as shown by the better migration and the higher proliferation of fibroblasts, and the thicker epithelium formed by the epithelial cells in the ELR-collagen foam, compared to the control collagen foam. Currently, we are testing ELR-collagen bilayer scaffolds, co-culturing fibroblasts and epithelial cells, for the reconstruction of full-thickness skin and oral mucosa equivalents.

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References

1. Rodríguez-Cabello JC, Prieto S, Reguera J, Arias FJ, Ribeiro A. Biofunctional design of elastin-like polymers for advanced applications in nanobiotechnology. J Biomater Sci Polym Ed. 2007;18:269–86.
2. Rodríguez-Cabello JC, Martín L, Alonso M, Arias FJ, Testera AM. "Recombinamers" as advanced materials for the post-oil age. Polymer. 2009;50:5159–69.
3. Chilkoti A, Christensen T, MacKay JA. Stimulus responsive elastin biopolymers: applications in medicine and biotechnology. Curr Opin Chem Biol. 2006;10:652–7.
4. Ozturk N, Girotti A, Kose GT, Rodríguez-Cabello JC, Hasirci V. Dynamic cell culturing and its application to micropatterned, elastin-like protein-modified poly(*N*-isopropylacrylamide) scaffolds. Biomaterials. 2009;30:5417–26.
5. Martínez-Osorio H, Juárez-Campo M, Diebold Y, Girotti A, Alonso M, Arias FJ, Rodríguez-Cabello JC, García-Vázquez C, Calonge M. Genetically engineered elastin-like polymer as a substratum to culture cells from the ocular surface. Curr Eye Res. 2009;34:48–56.
6. Betre H, Ong SR, Guilak F, Chilkoti A, Fermor B, Setton LA. Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. Biomaterials. 2006;27:91–9.
7. Martín L, Alonso M, Girotti A, Arias FJ, Rodríguez-Cabello JC. Synthesis and characterization of macroporous thermosensitive hydrogels from recombinant elastin-like polymers. Biomacromolecules. 2009;10:3015–22.
8. Nettles DL, Haider MA, Chilkoti A, Setton LA. Neural network analysis identifies scaffold properties necessary for in vitro chondrogenesis in elastin-like polypeptide biopolymer scaffolds. Tissue Eng A. 2010;16:11–20.
9. Urry DW, Pattanaik A, Xu J, Woods TC, McPherson DT, Parker TM. Elastic protein-based polymers in soft tissue augmentation and generation. J Biomater Sci Polym Ed. 1998;9:1015–48.
10. Adams SB Jr, Shamji MF, Nettles DL, Hwang P, Setton LA. Sustained release of antibiotics from injectable and thermally responsive polypeptide depots. J Biomed Mater Res B Appl Biomater. 2009;90:67–74.
11. Huang L, McMillan RA, Apkarian RP, Pourdeyhimi B, Conticello VP, Chaikof EL. Generation of synthetic elastin-mimetic small diameter fibers and fiber networks. Macromolecules. 2000;33:2989–97.
12. Garcia Y, Hemantkumar N, Collighan R, Griffin M, Rodríguez-Cabello JC, Pandit A. In vitro characterization of a collagen scaffold enzymatically cross-linked with a tailored elastin-like polymer. Tissue Eng A. 2009;15:887–99.
13. O'Brien FJ, Harley BA, Waller MA, Yannas IV, Gibson LJ, Prendergast PJ. The effect of pore size on permeability and cell

attachment in collagen scaffolds for tissue engineering. Technol Health Care. 2007;15:3–17.
14. Harley BA, Kim HD, Zaman MH, Yannas IV, Lauffenburger DA, Gibson LJ. Microarchitecture of three-dimensional scaffolds influences cell migration behavior via junction interactions. Biophys J. 2008;95:4013–24.
15. O'Brien FJ, Harley BA, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen-GAG scaffolds. Biomaterials. 2005;26:433–41.
16. MacNeil S. Progress and opportunities for tissue-engineered skin. Nature. 2007;445:874–80.
17. Sell SA, Francis MP, Garg K, McClure MJ, Simpson DG, Bowlin GL. Cross-linking methods of electrospun fibrinogen scaffolds for tissue engineering applications. Biomed Mater. 2008;3:045001.
18. Sisson K, Zhang C, Farach-Carson MC, Chase DB, Rabolt JF. Evaluation of crosslinking methods for electrospun gelatin on cell growth and viability. Biomacromolecules. 2009;10:1675–80.
19. Liang HC, Chang WH, Liang HF, Lee MH, Sung HW. Crosslinking structures of gelatin hydrogels crosslinked with genipin or a water-soluble carbodiimide. J Appl Polym Sci Symp. 2004;91:4017–26.
20. Badylak SF. Modification of natural polymers: collagen. In: Atala A, Lanza R, editors. Methods of tissue engineering. London: Academic Press; 2002. p. 505–15.
21. Zorlutuna P, Elsheikh A, Hasirci V. Nanopatterning of collagen scaffolds improve the mechanical properties of tissue engineered vascular grafts. Biomacromolecules. 2009;10:814–21.
22. McPherson DT, Morrow C, Minehan DS, Wu JG, Hunter E, Urry DW. Production and purification of a recombinant elastomeric polypeptide, G-(VPGVG)19-VPGV, from *Escherichia coli*. Biotechnol Prog. 1992;8:347–52.
23. Girotti A, Reguera J, Arias FJ, Alonso M, Testera AM, Rodríguez-Cabello JC. Influence of the molecular weight on the inverse temperature transition of a model genetically engineered elastin-like pH-responsive polymer. Macromolecules. 2004;37:3396–400.
24. Costa RR, Custódio CA, Testera AM, Arias FJ, Rodríguez-Cabello JC, Alves NM, Mano JF. Stimuli-responsive thin coatings using elastin-like polymers for biomedical applications. Adv Funct Mater. 2009;19:3210–8.
25. Faraj KA, van Kuppevelt TH, Daamen WF. Construction of collagen scaffolds that mimic the three-dimensional architecture of specific tissues. Tissue Eng. 2007;13:2387–94.
26. Vrana NE, Builles N, Justin V, Bednarz J, Pellegrini G, Ferrari B, Damour O, Hulmes DJ, Hasirci V. Development of a reconstructed cornea from collagen-chondroitin sulfate foams and human cell cultures. Investig Ophthalmol Vis Sci. 2008;49:5325–31.
27. Harley BA, Leung JH, Silva EC, Gibson LJ. Mechanical characterization of collagen-glycosaminoglycan scaffolds. Acta Biomater. 2007;3:463–74.
28. Kinikoglu B, Auxenfans C, Pierrillas P, Justin V, Breton P, Burillon C, Hasirci V, Damour O. Reconstruction of a full-thickness collagen-based human oral mucosal equivalent. Biomaterials. 2009;30:6418–25.
29. Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem. 2009;78:929–58.
30. Zhu X, Cui W, Li X, Jin Y. Electrospun fibrous mats with high porosity as potential scaffolds for skin tissue engineering. Biomacromolecules. 2008;9:1795–801.
31. Yannas IV, Lee E, Orgill DP, Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc Natl Acad Sci USA. 1989;86:933–7.
32. Sundararaghavan HG, Monteiro GA, Lapin NA, Chabal YJ, Miksan JR, Shreiber DI. Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence. J Biomed Mater Res A. 2008;87:308–20.
33. Rho KS, Jeong L, Lee G, Seo BM, Park YJ, Hong SD, Roh S, Cho JJ, Park WH, Min BM. Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing. Biomaterials. 2006;27:1452–61.
34. Bhardwaj N, Kundu SC. Electrospinning: a fascinating fiber fabrication technique. Biotechnol Adv. 2010;28:325–47.